Insight into the interfacial self-assembly and structural changes of hydrophobins

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CHAPTER 5

Unidirectional permeability of hydrophobin SC3 membrane formed at an oil/water interface

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Abstract

Hydrophobins are a class of small proteins that self-assemble into an amphipathic membrane at hydrophilic- hydrophobic interfaces and this process is crucial for the growth and development of fungi. Self-assembly of the SC3 hydrophobin of *Schizophyllum commune* at the water-air interface is accompanied with a number of consecutive conformational changes. Soluble state SC3 proceeds via the α-helical state and the β-sheet I state to the stable β-sheet II state, the latter is organized in amyloid-like fibrils. We here show that the SC3 hydrophobin also spontaneously self-assembles into the stable β-sheet II state at a water-oil interface, in a manner similar to that at the water-air interface. Porosity of the SC3 membrane was determined after overnight assembly at the water-oil interface. Molecules up to 10,000 Da freely passed the SC3 membrane from the oil to the water phase but molecules of 70,000 Da were excluded. In contrast, the SC3 membrane was impermeable for all molecules tested (MW ≥ 200) when they were dissolved in the aqueous phase. Thus, the SC3 membrane is unidirectionally permeable. A model explaining the asymmetric properties of the SC3 membrane is presented and the biological consequences are discussed.

Introduction

Hydrophobins are a class of small proteins that play an important role in fungal growth and development (Wösten, 2001). For instance, they allow fungi to escape an aqueous environment, confer hydrophobicity to fungal surfaces in contact with air, and mediate attachment of fungi to hydrophobic surfaces. The mechanism underlying these functions is based on the property of hydrophobins to self-assemble at any hydrophilic/hydrophobic interface into an amphipathic membrane. During self-assembly at the interface between the hydrophilic cell wall and a hydrophobic environment (the air or a hydrophobic solid), the hydrophilic side of the membrane will orient itself to the cell wall, while the hydrophobic side will be exposed. Aerial structures thus become water repellent, while hyphae in contact with a hydrophobic surface become attached.

Two different types of hydrophobins, class I and class II, have been distinguished on the basis of differences in their hydropathy patterns and biophysical properties (Wessels, 1994). SC3 of *Schizophyllum commune* is the best characterized class I hydrophobin. The eight conserved cysteine residues found in all hydrophobins (Wessels, 1997) form four disulfide bridges (de Vries et al., 1993). They prevent SC3 to spontaneously self-assemble in solution and account for the controlled assembly at hydrophilic-hydrophobic interfaces (de Vocht et al., 2000). SC3 contains 17-22 mannose residues that are attached to 12 threonine residues contained in the region preceding the first cysteine residue (de Vocht et al., 1998). These sugar residues are exposed at the hydrophilic side after self-assembly and thus determine the surface properties of this side (Wösten et al., 1994b; de Vocht et al.,1998; Scholtmeijer et al., 2002).

SC3 undergoes several conformational transitions during self-assembly. SC3 exists as oligomers in water (Wang et al., 2002), which are rich in β-sheet (de Vocht et al., 1998). Upon assembly at the water-air interface, SC3 proceeds via an intermediate form that has increased α-helical structure (α-helical state) to a stable end form that has increased β-sheet structure (β-sheet state) (de Vocht et al., 1998; 2002). SC3 in the β-sheet state initially has no clear ultrastructure ((β-sheet I state) (de Vocht et al., 2002) but after prolonged incubation the protein forms fibrils (β-sheet II state), called rodlets, which have a diameter of about 10 nm (Wösten et al., 1993). These rodlets have an amyloid-like nature. They are composed of two tracks consisting of 2-3 protofilaments that are 2.5 nm wide (Wösten and de Vocht, 2000). Moreover, like other amyloid fibrils (LeVine, 1993), they increase fluorescence of Thioflavin T and bind Congo-red (Wösten and de Vocht, 2000; Mackay et al., 2001; Butko et al., 2001). Assembly of SC3 at the Teflon surface is similar to that on the water-air interface. However, the protein can

only proceed from the intermediate α-helical state to the β-sheet state by treating the solid at elevated temperatures in the presence of detergent (de Vocht et al., 1998; 2002). Thus, SC3 does not spontaneously adopt the stable end form at this interface.

In contrast to the water-air and water-Teflon interface, self-assembly of SC3 was poorly characterized at the water-oil interface. We here show that self-assembly at this interface is similar to that at the water-air interface. Moreover, the porosity of assembled hydrophobin at the oil-water interface was determined, and, surprisingly, the membrane was found to be unidirectional permeable.

Results

Spontaneous SC3 membrane formation at a paraffin/buffer interface

Emulsions of organic solvents (butanol, hexane, and hexadecane) and oils (olive oil, paraffin) were prepared (2.5% v/v) by sonication, after which soluble-state SC3 was added immediately. The paraffin emulsion showed the highest stability (over weeks) as deduced from the turbidity of the aqueous phase. Dansyl-SC3 also stabilized the paraffin emulsion. When a sample was taken 30 min after adding SC3 to the oil emulsion, green fluorescent membranes were coating the paraffin droplets (Fig. 1A and C). A somewhat enhanced fluorescence was seen after overnight incubation. When a mixture of dansyl-SC3 (FRET donor) and dabcyl-SC3 (FRET acceptor) were used to stabilize the paraffin emulsion, fluorescence of dansyl SC3 was quenched at the oil-water interface (Fig. 1B and D), indicating that the membrane is composed of spatially proximate SC3 molecules.

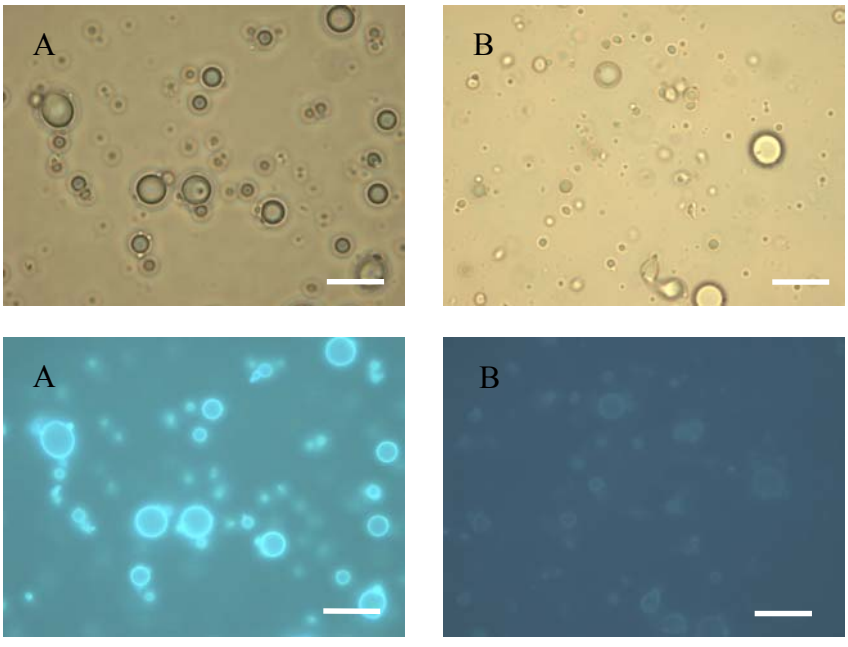

Fig. 1 Fluorescence of SC3 coated paraffin droplets in buffer. The upper pictures were illuminated with normal light; the lower ones with fluorescence light. Bar indicates 10 μm. A. dansyl-SC3 coated oil droplets. B. dansyl-SC3 (donor)/dabcyl-SC3 (acceptor) coated oil droplets.

Structural changes of SC3 upon assembly at a paraffin/buffer interface

The amyloid-specific fluorescent dye ThT has been shown to interact specifically with self-assembled SC3 in the β-sheet II state (Wösten and de Vocht, 2000; Butko et al., 2001), as illustrated in Fig. 2. Soluble state SC3 hardly interacts with ThT and thus little ThT fluorescence was observed. However, after vortexing (creating a large water-air interface) fluorescence was very strong, due to the conversion of soluble state SC3 into the β-sheet state. When colloidal Teflon was added to soluble state SC3, hardly any increase of fluorescence was observed. However, fluorescence of ThT was strongly increased after inducing the conversion of the intermediate α-helical state to the β-sheet state by treating the colloidal Teflon with hot detergent. Next, ThT fluorescence was studied after assembly of SC3 at the water-oil interface. Thirty min after adding soluble state SC3 to the paraffin emulsion, fluorescence had already increased above the value of soluble state SC3. This indicates that some SC3 had self-assembled into β-sheet-state structure (Fig. 2). After overnight incubation, approximately 40% of the maximum amount of aggregated β-sheet structure was formed as judged by the maximal level of ThT fluorescence for the same sample upon vortexing (Fig. 2). This experiment shows that SC3 spontaneously self-assembles at the oil-water interface into the stacked β-sheet state. Thus, self-assembly is not arrested in an intermediate state, like observed in case of the water-Teflon interface.

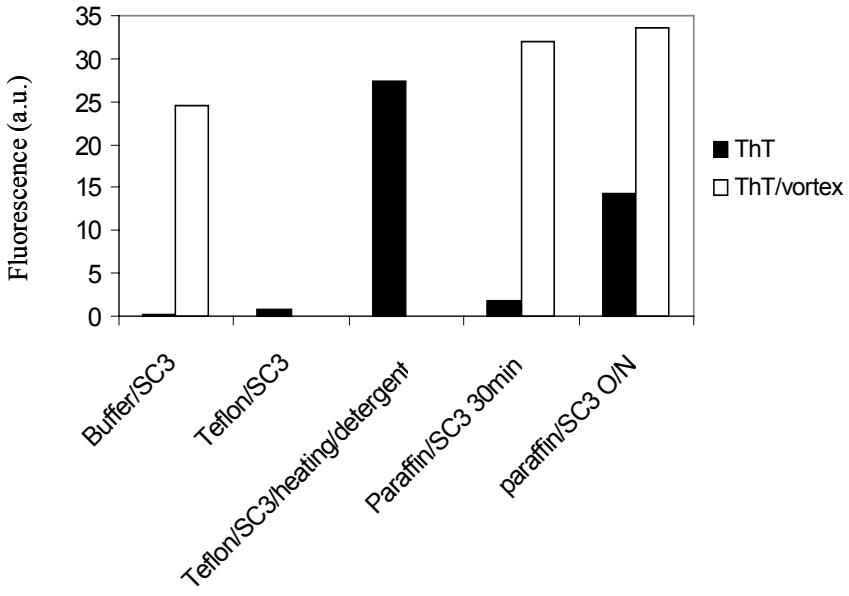

Fig. 2 Amyloid-like β-sheet-state formation of SC3 at the water-air, water-oil and water-Teflon interface, as determined by the increase in ThT fluorescence. Filled and open columns represent samples before and after vortexing for 5 min, respectively

Unidirectional permeability of the SC3 membrane formed at the oil-water interface

To determine the permeability of the SC3 membrane, the hydrophobin was allowed to self-assemble at a paraffin-water interface in a cuvette. A downward curved interface was formed immediately after adding the paraffin oil on top of the SC3 solution, whereas, without SC3, it formed a flat surface. The permeability of the SC3 membrane to solutes of various masses was then measured. The kinetics of diffusion of fluorescent marker molecules, that are soluble in both water and paraffin, was determined from either side of the membrane by following the fluorescence change in the buffer phase. First, diffusion of pyrene (MW 202) was followed. To exclude the possibility that the SC3 hydrophobin changes the fluorescence properties of the marker, the fluorescence emission spectrum was monitored in the absence and presence of SC3. Pyrene excited at 345 nm showed an emission spectrum composed of two bands, a "monomer band" with peaks near 390 nm and a "dimer band (excimer band)" with a broad peak near 460 nm. The excimer to monomer ratio, which can be calculated from the fluorescence intensities at 460 and 390 nm, has been used to report the environmental hydrophobicity once pyrene accesses the interior of micelles, membranes or proteins (Sen et al., 1990; Bhattacharyya et al., 2002). When pyrene would bind to a hydrophobic environment in the SC3 molecule, a significant change in fluorescence intensities at 390 and 460 nm would have been expected. As shown in Fig. 3A, the pyrene excimer to monomer ratio decreased slightly in time in the presence of SC3, indicating that a large amount of pyrene monomers (at least 90%) still remained in solution even after overnight incubation. Therefore, the changes in pyrene fluorescence intensity (as measured at 395 nm; see below) in the presence of SC3 are not due to binding of pyrene to the protein but rather to diffusion of the marker across the hydrophobin membrane. This conclusion was confirmed by another control experiment in which the amount of pyrene was accumulated (see the paragraph after next) in the SC3/buffer phase in a paraffin/SC3/buffer sample independent of SC3 concentrations (varied from 50 to 200 μg/ml) used, indicating that pyrene does not bind to soluble-state significantly.

Pyrene rapidly diffused from buffer to paraffin in the absence of SC3 (Fig. 3B). When the diffusion experiment was started 30 min after assembling SC3 at the paraffin-buffer interface, passage of the marker was reduced. However, it was completely blocked after overnight assembly of the hydrophobin (Fig. 3B). Some diffusion was observed when the temperature was raised to 50-80 °C (data not shown). In contrast, pyrene still diffused at room temperature from paraffin to the buffer phase after overnight assembly of SC3 (Fig. 3C), although it was reduced compared to an interface without hydrophobin. As a control, SC3 was replaced for BSA and

lysozyme (100 μg/ml). When pyrene was added after overnight incubation of the samples, diffusion of the molecule from buffer to paraffin was only 20% reduced in 2.5 h (data not shown).

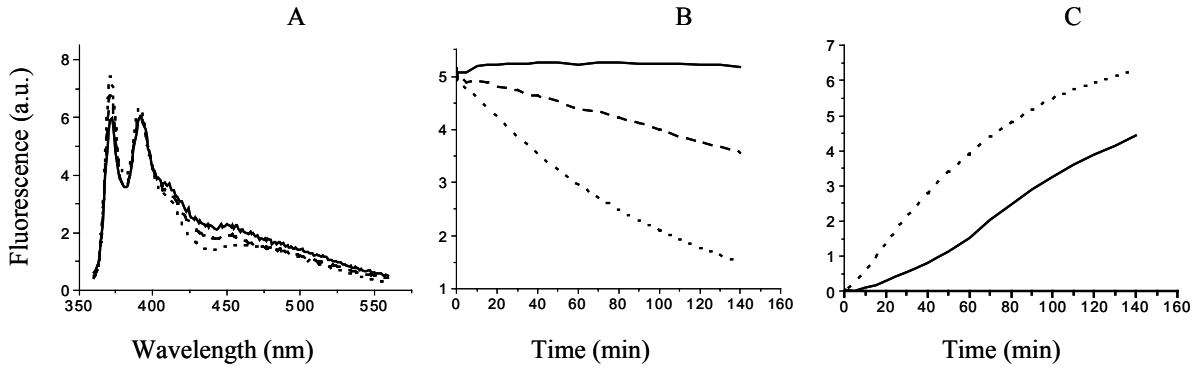

Fig. 3 Pyrene diffusion through the SC3 membrane formed at the buffer-paraffin interface. (A) Pyrene fluorescence is only slightly changed in the presence of SC3. Fluorescence spectrum of pyrene in buffer (dotted line), after incubation for 30 min (dashed line) and 16 h (solid line) in buffer with 100 μg/ml of soluble state SC3. (B) Pyrene diffusion from buffer to paraffin in the absence of SC3 (dotted line) or in the presence of an SC3 membrane formed during 30 min (dashed line) or 16 h (solid line). (C) Pyrene diffusion from paraffin to buffer in the absence of SC3 (dotted line) or in the presence of an SC3 membrane formed during overnight incubation from a solution containing 100 μg/ml of protein (solid line).

In another experiment, a paraffin/buffer (control) and a paraffin/SC3/buffer sample were incubated overnight as described above. Subsequently, 50 μl of paraffin/pyrene solution was carefully added on top of the paraffin phase of each sample, and the pyrene fluorescence in the buffer phase was monitored (Fig. 4). For both samples, the pyrene fluorescence intensity reached a constant value within 2.5 h, but the amount of pyrene in the buffer phase of the paraffin/SC3/buffer sample reached a level about 2 times as high as that of the control sample, indicating that pyrene was accumulated in the buffer phase in the presence of the SC3 membrane beyond the level expected on the basis of its partition coefficient. Obviously, the unidirectional permeability of the SC3 membrane resulted in a block of pyrene back-diffusion to paraffin, only allowing its one-way diffusion into buffer.

The formation of a SC3 membrane with unidirectional permeability was protein concentration dependent. Pyrene diffusion from buffer to paraffin was not completely blocked when 10 μg/ml of SC3 was used instead of 100 μg/ml to form the membrane at the paraffin/buffer interface during overnight incubation (Fig. 5). This was also not the case when pyrene diffusion was

measured 1 week after assembly at the interface was started (data not shown). The effect was even more dramatic when 3 μg/ml of SC3 was used. Pyrene diffusion from buffer to paraffin was almost identical to that of the control sample in the absence of SC3 (compare Fig. 5, dashed line and dash-dotted line). Apparently, this concentration was too low to allow formation of a (complete) hydrophobin membrane.

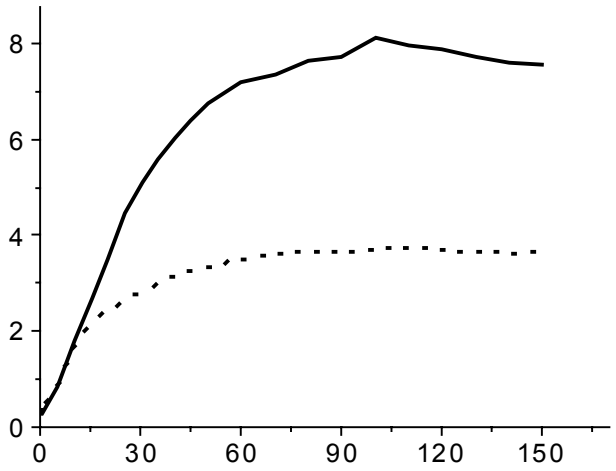

Fig. 4 Accumulation of pyrene in the buffer phase, using an overnight-incubated paraffin/SC3/buffer system. 50 μl of saturated pyrene/paraffin solution was added carefully to the top of the system, and the pyrene fluorescence was determined in time in the buffer phase. Solid line, the sample in the presence of the SC3 membrane. Dotted line, the control sample in the absence of the SC3 membrane.

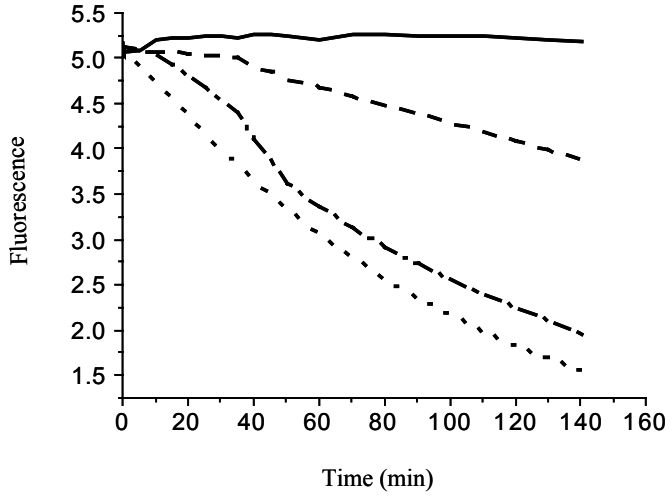

Fig. 5 Diffusion of pyrene from buffer to paraffin depends on the concentration of soluble state SC3 in the starting solution. Permeability was measured after overnight assembly using a SC3 concentration of 100 μg/ml (solid line), 10 μg/ml (dashed line); and 3 μg/ml (dash-dotted line). A solution in the absence of SC3 served as a control (dotted line).

The size exclusion limit of the SC3 membrane was further characterized with fluorescent-labeled neutral dextran. Dextran 3,000 easily equilibrated between paraffin and buffer in the absence of a SC3 membrane. The kinetics of diffusion were approximately the same in both directions (compare dotted lines in Fig. 6A and B). In contrast, in the presence of a SC3 membrane Dextran 3,000 diffused slowly from paraffin to buffer (Fig. 6A), but diffusion was

completely blocked in the reverse direction (Fig. 6B). Dextran 10,000 and 70,000 diffused more slowly from paraffin to buffer and, therefore, a higher temperature (50°C) was used to accelerate the diffusion process. Dextran 10,000 behaved similar to pyrene and Dextran 3,000; the diffusion from paraffin to buffer was retarded by the SC3 membrane, whereas the diffusion from buffer to paraffin was blocked (Fig. 6C and D). The passage of large molecules through the membrane from its hydrophobic side was even confirmed by replacing Dextran with cytochrome-c, a globular protein with molecular weight of about 12,000 Da (data not shown). The diffusion of Dextran 70,000 from paraffin to buffer, however, was totally blocked when a SC3 membrane was present (data not shown). This indicates that the size exclusion limit of the hydrophobic side of the membrane is between 10,000 and 70,000 Da. Diffusion of Dextran 70,000 from buffer to paraffin was too slow to be determined in this case, for both control and SC3 samples.

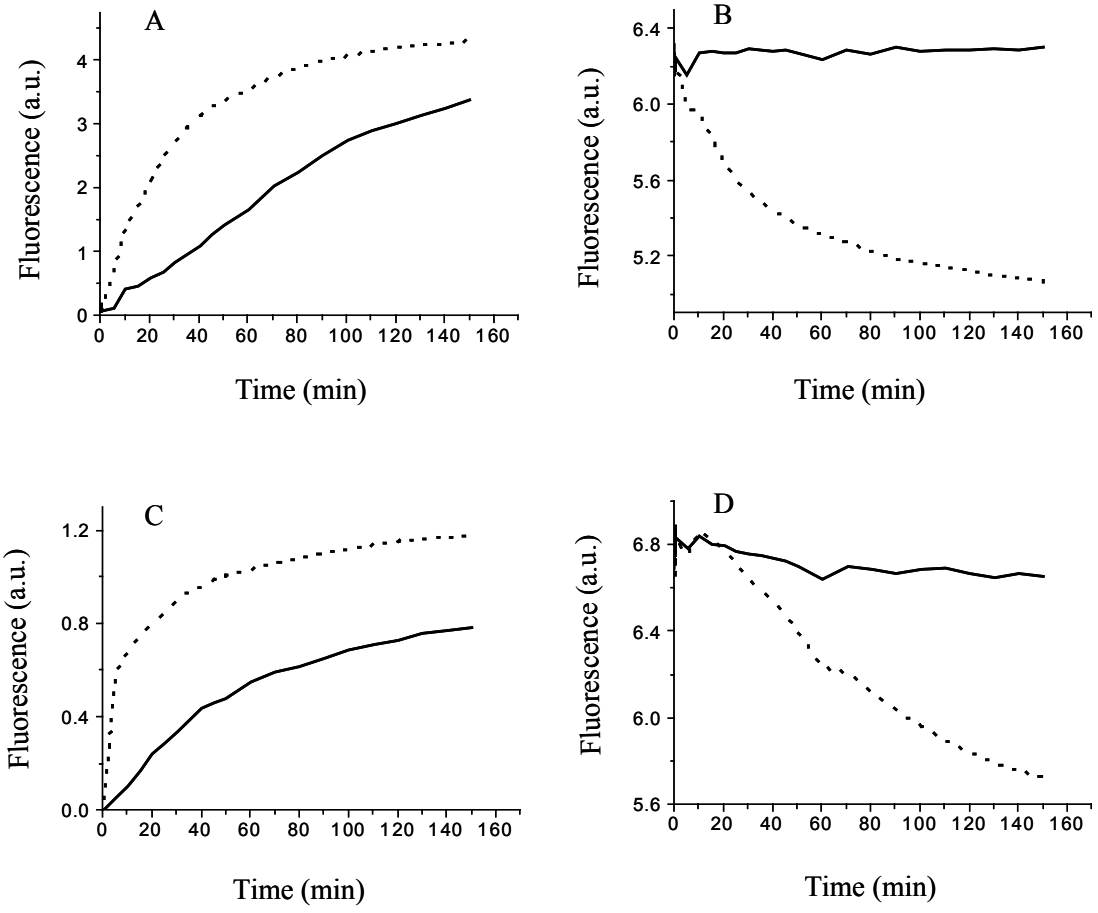

Fig. 6 Diffusion of Dextran 3,000 (A and B) and Dextran 10,000 (C and D) in the absence (dotted lines) and presence (solid lines) of a SC3 membrane formed during overnight incubation at the paraffin/buffer interface using a protein solution of 100 μg/ml. Diffusion was measured from paraffin to buffer (A and C) and from buffer to paraffin (B and D).

The diffusion data of the fluorescent marker molecules were fit to an one-exponential equation. The permeability properties of the SC3 membrane in terms of molecule diffusion-rate constants are summarized in Table 1. Clearly, the presence of the SC3 membrane retarded diffusion of all the marker molecules from paraffin to buffer but diffusion was totally blocked from the other direction.

Discussion

We have shown that self-assembly of the hydrophobin SC3 at the water-oil interface is very similar to that at the air-water interface. The hydrophobin spontaneously self-assembles into amyloid-like β-sheet stacks (β-sheet II state) without being arrested in an intermediate state as observed at the Teflon-water interface. This finding offered us the unique possibility to study the permeability of the hydrophobin membrane as also formed at surfaces of fungal structures.

Dansyl-labeled SC3 stabilized paraffin droplets by forming a fluorescent coating. The fluorescence of these molecules was efficiently quenched by FRET when a mixture of dansyl-SC3 (donor) and dabcyl-SC3 (acceptor) was used to coat the oil droplets. From this experiment it can be concluded that donor and acceptor were in close proximity, which is typical for the β-sheet state and not for the α-helical state (Wang et al., 2002). Further evidence that SC3 spontaneously adopted the β-sheet state and formed amyloid like fibrils (β-sheet II state) at the oil-water interface was obtained by studying the assembly with ThT. Increased fluorescence of this dye is associated with the formation of amyloid-like rodlets (Wösten and de Vocht, 2000; Butko et al., 2001; Stroud et al., 2003). ThT fluorescence increased significantly when SC3 was added to a paraffin emulsion, albeit to a lesser extent than at the water-air interface. Probably, part of the SC3 was still in the soluble state and not associated with oil droplets (Wösten et al., 1994a). The increased fluorescence of ThT is in good agreement with a previous study (Wösten et al., 1994a), showing the amyloid-like rodlets at the oil-water interface after freeze-fracturing SC3-coated oil droplets. However, at that time it could not be excluded that these rodlets were first formed at the water-air interface (due to sonication) and then associated with the oil droplets.

Using fluorescent molecules with different molecular weights, it was shown that the SC3 membrane in the β-sheet II state is unidirectional permeable. Molecules up to 10,000 Da could pass the membrane from the hydrophobic side (paraffin), but not in the reverse direction. The fact that pyrene accumulated to a higher extent in the aqueous phase in the presence of a SC3 membrane confirms this conclusion. Formation of the unidirectional permeable membrane was

Table 1. Directional permeability of the SC3 membrane formed at a paraffin/buffer interface. The rate constants (min^{-1}) of diffusion of various marker molecules were obtained by fitting the data to the equation $y=y0 + A \times e^{-x*}$, with A representing diffusion rate constant (min^{-1}).

		pyrene	Dextran 3000 (neutral)	Dextran 10000 (neutral)	Dextran 70000 (neutral)
Diffusion paraffin to buffer 2.5 h	No SC3 membrane	6.37 ± 0.04	4.19 ± 0.05	1.96 ± 0.06	< 0.01 (2.24 ± 0.02)*
	SC3 membrane	4.52 ± 0.06	3.68 ± 0.13	0.82 ± 0.01	< 0.01 (0.11 ± 0.005)*
Diffusion buffer to paraffin 2.5 h	No SC3 membrane	5.00 ± 0.07	1.12 ± 0.02	< 0.01 (2.45 ± 0.60)*	n.d.
	SC3 membrane	0.10 ± 0.01	0.07 ± 0.02	< 0.01 (0.20 ± 0.02)*	n.d.

* experiments were done at 50°C instead of room temperature in order to accelerate the diffusion of marker molecules.

protein concentration dependent. It was formed at 100 μg/ml but not at 3 or 10 μg/ml. At the lowest concentration diffusion of marker molecules was not hampered at all, indicating the absence of a membrane. This may be due to the fact that assembly of SC3 depends on a critical concentration (3.7 μg/ml; de Vocht et al., 2001). A concentration of SC3 in the aqueous phase of 10 μg/ml is sufficient for self-assembly. However, at this concentration rodlets could not be observed at the water-air interface although a discrete SC3 membrane was formed (de Vocht et al., 2002). This suggests that at such a low concentration SC3 is arrested in the β-sheet I state. The secondary structure of this state cannot be discriminated from that of the β-sheet II state but the ultrastructure is clearly different, i.e., a featureless film versus a rodlet decorated film (de Vocht et al., 2002). This difference in organization may affect the permeability characteristics.

Based on previous results and data presented in this paper we propose a model explaining the unidirectional permeability of the SC3 membrane (Fig. 7). During self-assembly, a part of the SC3 molecule (C40-C73; called the 2nd loop) orients itself to the hydrophobic phase of the interface (Wang et al., unpublished), while both the N- and C-terminus become exposed to the hydrophilic phase (Wösten et al., 1994b; Wang et al., 2002; Wang et al., unpublished). SC3 could assemble at the interface in such a way that the space between the protein units on the hydrophobic side is sufficient to allow large molecules to pass, whereas it is limited on the hydrophilic side due to the hydrophilic N-and C-terminal stretches. The long N-terminus and short C-terminus could form a network and act as a "valve". The uni-directionality is reduced at high temperature, probably because of increased flexibility of the N- and C-terminal portions and weaker intermolecular interactions.

The unidirectional permeability of the SC3 membrane does not only have consequences for the use of these molecules in medical and technical applications (Wessels, 1997; Scholtmeijer et al., 2001), it also has important physiological implications. The SC3 membrane covers aerial hyphae and hyphae in contact with a hydrophobic substrate such as that of a plant (Wösten et al., 1994a,c). This membrane would allow passage of molecules from the environment with molecular weights up to at least 10,000 Da. Thus, the hydrophobin would not physically prevent uptake of nutrients such as mono- and oligosaccharides and peptides. On the other hand, molecules in the cell wall, even when they are very small, would not be able to diffuse into environment. Sealing the hyphal surface with a hydrophobin membrane would enable the fungus to effectively store reserve molecules in the cell wall that could be used for instance during spore germination. Also, the diffusion of molecules that elicit plant defence may be prevented (Cole and Kirkland, 1991; Wösten and Wessels, 1997). The hydrophobin would thus contribute to

infection of fungal plant pathogens. The results imply that enzymes would also not be able to diffuse into the environment once a hydrophobin membrane has assembled. These enzymes are crucial for fungal growth since they degrade polymeric substrates into small molecules that can be taken up and serve as nutrients.

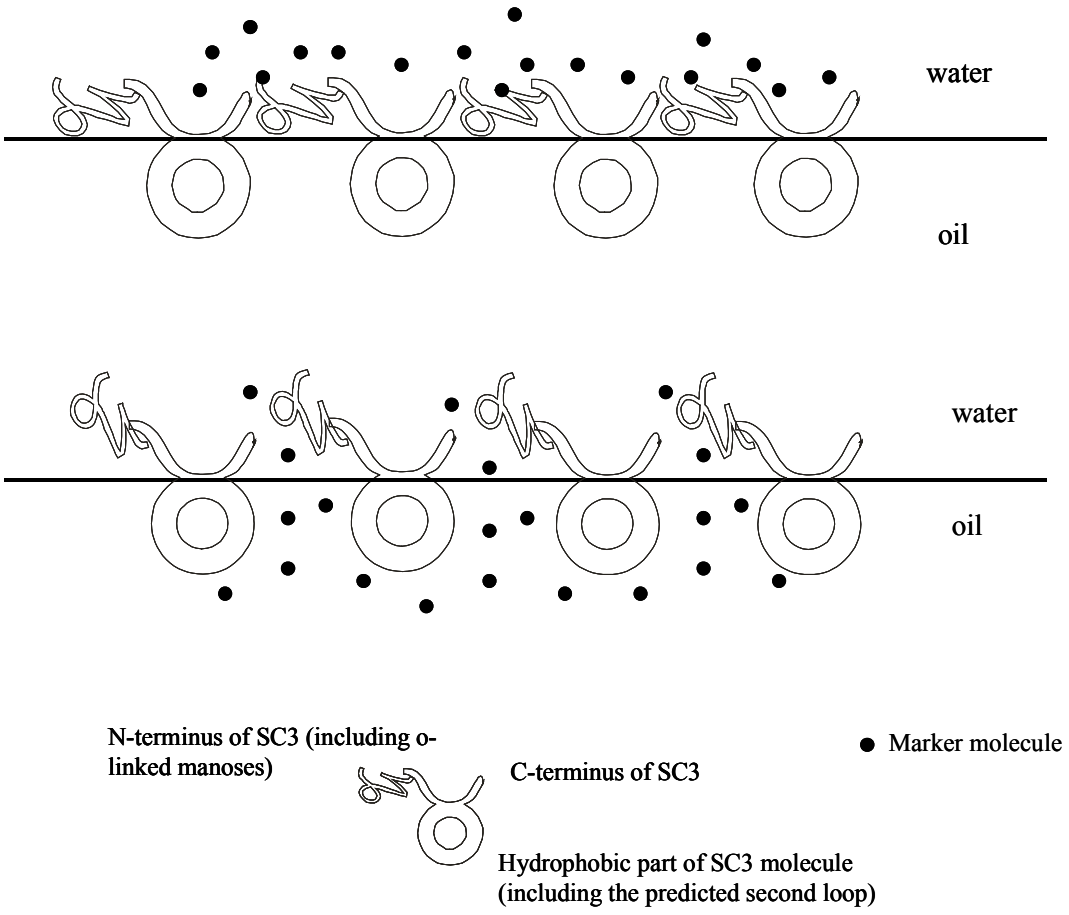

Fig. 7 A model for the arrangement of SC3 molecules at an oil/water interface, resulting in unidirectional permeability of the membrane. The N-terminal and C-terminal parts of SC3 that reside at the hydrophilic side of the SC3 membranes prevent diffusion from the aqueous phase but not from the hydrophobic phase.

Enzymes are only secreted at the extreme tip of growing hyphae (Wösten et al., 1991, 1994c; Moukha et al., 1993) and this part is expected not to be coated with a discrete hydrophobin membrane. This is explained by the fact that the cell wall at the extreme tip is very different from that at subapical parts of the hyphae (Wessels, 1984; 1990). The subapical cell wall is a relatively static structure due to extensive cross-linking of cell wall polymers. A SC3 membrane at these parts of the hyphae is not exposed to stretch forces. The cell wall at the extreme tip of a growing hypha, on the other hand, is a highly dynamic structure (Wessels, 1984; 1990). There is a flow of

secreted proteins and cell wall polymers, that are not yet cross-linked, from the inner part of the cell wall to the outer surface. This flow stretches the outer surface of the tip continuously. It not only enables extension of the hyphae but would also rupture hydrophobin assemblages at this site. Thus, the hyphal tip of growing hyphae would not be sealed with a continuous hydrophobin membrane, allowing diffusion of molecules such as enzymes. Once growth of the hyphae stops, the flow of proteins and cell wall polymers ceases. The cell wall at the apex will be cross-linked like that at subapical parts and a continuous hydrophobin film can be formed. As a result, the cell wall of a non-growing hyphae (or spore) is completely sealed, preventing loss of cell wall molecules into the environment.

Materials and methods

Purification and labeling of hydrophobins

A monokaryotic strain of *S. commune* with a deleted *SC15* gene (Lugones, 1999) was used for production of the SC3 hydrophobin. The fungus was grown in production medium in 1 L shaken cultures (225 rpm) for 5 – 7 days at 24 °C (Scholtmeijer et al., 2002). SC3 was purified from the culture medium as described (Wösten et al., 1993; Wessels, 1997) and labeled with dansyl and dabcyl according to Wang et al. (2002).

Self-assembly of SC3 at the water-oil and water Teflon interface

Freeze dried SC3, either or not labelled with dabcyl or dansyl, was treated with TFA to dissociate assemblages into soluble state SC3 (Wösten et al., 1993). After removing the solvent by a stream of air, the protein was taken up in 50 mM sodium phosphate, pH 7.0 (1 mg/ml). This stock solution was made fresh and used, unless otherwise indicated, at a final concentration of 100 μg/ml. In case of FRET experiments, stock solutions of dabcyl and dansyl labelled SC3 were mixed 1:1 and incubated for 30 minutes before use. Oil/buffer stocks were prepared by mixing paraffin and buffer (50 mM sodium phosphate, pH 7.0) in a ratio (v/v) of 2.5:100. The mixture was bath sonicated for 10 min just before adding the hydrophobin. Colloidal Teflon was prepared as described (de Vocht et al., 1998) and amounts were added to hydrophobin solutions such that the protein could cover maximally 10% of the Teflon surface (Wösten et al., 1994a). By adding colloidal Teflon to a SC3 solution, the protein adopts the α-helical state. The β-sheet-state was attained by heating the mixture for 30 min at 65°C in the presence of 0.1% Tween 80.

Thioflavin T fluorescence of SC3 at a hydrophobic-hydrophilic interface

A Thioflavin T (ThT) stock solution (300 μM) was added 1:100 at fixed time intervals to mixtures of oil and water and Teflon and water to which hydrophobin was added. ThT fluorescence was measured using a SPF-500C spectrofluorometer (SLM Aminco). The excitation wavelength used was 450 ± 4 nm, and the emission intensity was measured at 500 ± 4 nm. Data collected for each sample were corrected for the signal before the addition of ThT.

Permeability of the SC3 membrane formed at a paraffin oil/buffer interface

The following fluorescent compounds were used to study the permeability of the SC3 membrane; pyrene (MW 202.26 Da), neutral Texas Red Dextran (MW 3,000 Da), and neutral rhodamine B Dextran (MW 10,000 Da and 70,000 Da). Pyrene was purchased from Fluka, the other compounds from Molecular Probes. Sample preparation and measurements were done in a 1cm × 1cm (4 ml) quartz cuvette with a stirring bar at the bottom. For pyrene, an excitation wavelength of 347 nm and an emission wavelength of 395 nm were used. For Texas red-labelled dextran, the excitation wavelength was 582 nm, and the emission wavelength 610 nm. For Rhodamine B labeled dextran, these wavelengths were 570 nm and 603 nm, respectively. The change of fluorescence intensity in the buffer phase was usually followed for 2.5 hours, with a SPF-500C spectrofluorometer (SLM Aminco). For this, the cuvette was placed in the spectrofluorometer in such a way that only the buffer phase was situated in the light path. The slit width for both excitation and emission was 4 nm. Diffusion experiments were carried out at room temperature, except those for dextran 10,000 and dextran 70,000, which were done at 50°C to accelerate the diffusion.

Diffusion from paraffin to buffer. A cuvette was filled with 2 ml of a solution of 100 μg/ml soluble-state SC3 on top of which 1 ml of paraffin oil was placed. The cuvette was left at room temperature overnight to allow SC3 assembly at the water-oil interface. The cuvette was then placed in the holder of the spectrofluorometer. The diffusion experiment was started by carefully adding 1 ml of paraffin containing one of the fluorescent markers at a saturating concentration on top of the original paraffin layer. The stirring-bar was then immediately set to slow rotation. The control experiment was done in the same way except that there was no SC3 present in the buffer phase.

Diffusion from buffer to paraffin. A cuvette was filled with 2 ml of a solution of 100 μg/ml soluble-state SC3 supplemented with one of the fluorescent markers at a saturating

concentration. One ml of paraffin containing the same fluorescent marker at a saturating concentration was then carefully added on top of the SC3 solution. After overnight or 30 min assembly at the water-oil interface at room temperature, the cuvette was placed in the holder of the fluorescence spectrofluorometer. To start the diffusion experiment, 0.5 ml of paraffin was carefully removed from the incubated sample, and replaced by 1.5 ml of the oil lacking the marker. Measurements were immediately started under conditions of slow stirring. The control experiment was done in the same way except that there was no SC3 present in the buffer.